AD_____

CONTRACT NO: DAMD17-87-C-7014

TITLE: PRODUCTION OF ANTIGENS AND ANTIBODIES FOR DIAGNOSIS OF ARBOVIRUS DISEASES

PRINCIPAL INVESTIGATOR: Robert E. Shope, M.D.

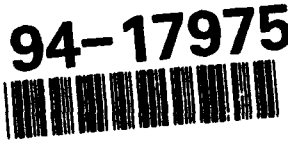
CONTRACTING ORGANIZATION: Yale University School of Medicine
333 Cedar Street
P.O. Box 20846
New Haven, Connecticut 06510-8047

REPORT DATE: May 20, 1994

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research, Development, Acquisition and Logistics Command (Provisional), Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited



REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	20 May 1994	Annual Report (4/1/87 - 3/31/91)

4. TITLE AND SUBTITLE
Production of Antigens and Antibodies for Diagnosis of Arbovirus Diseases

5. FUNDING NUMBERS
Contract No.
DAMD17-87-C-7014

6. AUTHOR(S)
Robert E. Shope, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University School of Medicine
333 Cedar Street
P.O. Box 20846
New Haven, Connecticut 06510-8047

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research, Development, Acquisition and Logistics Command (Provisional), Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Reagents were prepared for diagnosis by ELISA of 54 arthropod-borne viruses that cause human disease and are of potential or actual military importance. Mouse brain antigens made by the sucrose-acetone technique to the 54 arboviruses will be useful for assay of arboviral IgG and IgM and may also, in some instances, be used in hemagglutination-inhibition tests. The antigens were inactivated by beta-propiolactone. Rabbit IgG, purified by ammonium sulfate concentration, was prepared to 20 of the arboviruses and was designed to use to capture antigen in ELISA. The titers varied considerably from one rabbit to another, but most may be used in an optimal dilution of 1:1000 to 1:32000.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Antigens, Antibodies, Arbovirus, Enzyme linked immunosorbent assay	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

SUMMARY

Reagents were prepared for diagnosis by ELISA of 54 arthropod-borne viruses that cause human disease and are of potential or actual military importance. Mouse brain antigens made by the sucrose-acetone technique to the 54 arboviruses will be useful for assay of arboviral IgG and IgM and may also, in some instances, be used in hemagglutination-inhibition tests. The antigens were inactivated by beta-propiolactone. Rabbit IgG, purified by ammonium sulfate concentration, was prepared to 20 of the arboviruses and was designed to use to capture antigen in ELISA. The titers varied considerably from one rabbit to another, but most may be used in an optimal dilution of 1:1000 to 1:32000.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

Robert E. Shope May 20, 1994

PI Signature Date

TABLE OF CONTENTS

BODY OF REPORT

1. STATEMENT OF THE PROBLEM UNDER STUDY

The problem under study is the development of ELISA systems for rapid serological diagnosis of human arboviral diseases of military importance. These systems will be applicable in the field for sensitive, specific, and rapid diagnosis of arboviral infections of both civilian and military populations.

2. BACKGROUND AND REVIEW OF APPROPRIATE LITERATURE AND/OR EARLIER REPORTS

The enzyme-linked immunosorbent assay (ELISA) was devised by E. Engvall and P. Perlmann in 1971 (Enzyme-linked immunosorbent assay. Quantitative assay of immunoglobulin G. Immunochemistry 8:871-874). An antigen or antibody is conjugated to an enzyme allowing quantitative assay when a substrate is added and the reaction is read by color change. The test for the purposes of this project is adapted for detection of an antibody rise in acute and convalescent sera. ELISA is extremely sensitive and does not require the more elaborate equipment needed for the immunofluorescence and radio immune assays. The initial adaptation of ELISA to arboviruses was accomplished at Yale (Frazier, C.L. and Shope, R.E. 1979. Detection of antibodies to alphaviruses by enzyme-linked immunosorbent assay. J. Clin. Microbiol. 11:564-551).

For the detection or capture of antigen, the ELISA requires purified antibody to coat the solid phase and bind the virus. The sensitivity of antibody detection is improved by addition of an initial coating antibody to the ELISA system without increasing background reaction (Yolken, R.H. and Stopa, P.J. 1980. Comparison of seven enzyme immunoassay systems for measurement of cytomegalovirus. J. Clin. Microbiol. 11:564-551). In addition it is relatively easy and inexpensive to purify the antibody and relatively more difficult to purify the antigen. Using antibody as coat permits the use of sucrose-acetone processed arboviral antigens which are standard antigens in general use for routine hemagglutination-inhibition and complement fixations tests. Immune rabbit serum has proved satisfactory when purified by ammonium sulfate precipitation. Also, by developing a coating antibody other than mouse (i.e. rabbit) one can use the large number of standardized mouse brain antigens and mouse ascitic fluids available in the reference collections of Yale, CDC, and military laboratories.

3. RATIONALE USED IN CURRENT STUDY

The ELISA used in the current study utilized a solid-phase to which was attached semi-purified rabbit anti-arbovirus IgG. This IgG captured homologous arboviral antigen which, in turn, reacted with human or other species antibody. The indicator system was anti-species (e.g. anti-human) antibody to which was conjugated the enzyme, peroxidase. The peroxidase was detected by the substrate ABTS. In order to prepare the rabbit anti-arbovirus IgG, it was necessary first to try to adapt each arbovirus to rabbit tissue culture, RK-13, so that there would be a homologous species of tissue in the immunogen injected into the rabbit. Otherwise, the immune rabbit sera would have been expected to contain antibodies to the heterologous tissue used to immunize.

4. EXPERIMENTAL METHODS

a. The 54 viruses used in the development of the ELISA were as follows:

Bandia	Hazara	Rocio
Bangui	Hughes	Ross River
Belterra	Ilesha	Sagiyama
Bhanja	Ilheus	Semliki Forest
Bunyamwera	Inkoo	Sindbis
Bussuquara	Jamestown Canyon	Snowshoe hare
Bwamba	Japanese encephalitis	Tahyna
Cache Valley	LaCrosse	Tataguine
Candiru	Lymphocytic choriomeningitis	Tensaw
Catu	Maguari	Tick-borne encephalitis
Chagres	Mayaro	Toscana
Chandipura	Mucambo	VSV-Indiana
Cocal	O'nyong-nyong	VSV-New Jersey
Colorado tick fever	Oriboca	West Nile
Dugbe	Oropouche	Sicilian sandfly fever
Ganjam	Piry	Zika
Germiston	Qalyub	Shuni
Guaroa	Quaranfil	Catu

b. Viruses were passaged intracerebrally in baby mice. When the mice sickened, brains were harvested and antigen was prepared by the sucrose-acetone technique of Clarke and Casals (Clarke, D.H. and Casals, J. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg. 7:561-573). Two hundred fifty milliliters of each antigen were produced. The antigens were rehydrated in pH 9.0 tris buffer 0.1M, then infectivity was inactivated by addition of 0.1 % beta-propriolactone excepting for the alphaviruses which were inactivated with 0.3 % beta-propriolactone. The beta-propriolactone/antigen mixture was held for 3 days at 4C, then stored frozen at -70C.

c. Viruses were passaged in a rabbit cell line, RK-13, and observed for cytopathic effect. Those that did not have cytopathic effect were subjected to sub-culture in Vero cells or were tested for antigen content by lysing washed cells and carrying out ELISA by direct coating of the solid phase with the cell lysate. For those that had either infectious virus or antigen (indicating that the virus was replicating), tissue culture fluid was used to immunize rabbits.

d. Rabbits were inoculated with virus in the lateral ear vein at weekly intervals for 3 inoculations. One week later blood was taken and the serum tested by ELISA for its capacity to capture homologous antigen. Rabbits were boosted if needed. When satisfactory titer of antibody was obtained, the rabbits were bled and euthanized. An attempt was made to obtain 300 ml of rabbit serum for each virus. Rabbit sera were subjected to ammonium sulfate precipitation to purify IgG (Hebert, G.A., Pelham, P.L., and Pittman, B. 1973. Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse, and goat antisera. Appl. Micro. 25:26-36). Ammonium sulfate treated sera were stored at -20C.

e. ELISA was done by the technique of Meegan et al. (Meegan, J.M., Yedloutschnig, R.J., Peleg, B.A., Shy, J., Peters, C.J., Walker, J.S., and Shope, R.E. 1985. An enzyme immunoassay for detection of antibodies to Rift Valley fever virus in ovine and bovine sera. Am. J. Vet. Research 48:1138-1141).

5. RESULTS

a. Production of antigens:

Inoculation of mice and harvesting of brains for antigen production was completed with the 54 arboviruses. Sucrose-acetone extraction of mouse brains and/or crude brain extraction of the following viruses was completed (liver was extracted in some cases):

Virus	Approximate volume (ml) Sucrose-acetone antigen	Approximate volume crude brains/livers
Bandia	284	
Bangui	-	150
Belterra	239	
Bhanja	237	
Bunyamwera	269	
Bussuquara	-	78
Bwamba	287	
Cache Valley	-	66
Candiru	-	66
Catu	-	25
Chagres	97	
Chandipura	310	
Cocal	-	53
Colorado tick fever	-	107
Dugbe	259	
Ganjam	67	100
Germiston	473	
Guaroa	-	133
Hazara	266	
Hughes	280	
Ilesha	213	
Ilheus	305	
Inkoo	-	125
Jamestown Canyon	-	113
Japanese encephalitis	278	27
LaCrosse	13	130
Lymphocytic choriomeningitis	-	100
Maguari	268	
Mayaro	229	
Mucambo	-	125
O'nyong-nyong	375	
Oropouche	241	
Oriboca	23	60
Piry	-	30
Qalyub	338	
Quaranfil	279	35
Rocio	87	100
Ross River	256	
Sagiyama	-	125
Salehabad	85	
Semliki Forest	182	
Shuni	-	73

Virus (continued)	Approximate volume (ml) sucrose-acetone antigen	Approximate volume crude brains/livers
Sicilian sandfly fever	37	
Sindbis	40	55
Snowshoe hare	306	
Tahyna	-	35
Tataguine	280	
Tensaw	-	125
Tick-borne encephalitis	200	37
Toscana	349	
VSV-Indiana	271	
VSV-New Jersey	422	
West Nile	25	45
Zika	501	

b. Adaptation of arboviruses to growth in RK-13 rabbit cells:

This was the most challenging aspect of the project from a technical viewpoint. Although initial trials were promising, many of the arboviruses did not replicate, or if they adapted, the tissue culture fluids did not immunize well. The following viruses were satisfactory:

Bunyamwera
Bussuquara
Bwamba
Chandipura
Cocal
Germiston
Hazara
Ilheus
Jamestown Canyon
Japanese encephalitis
Mayaro
Mucambo
Piry
Qalyub
Quaranfil
Ross River
Semliki Forest
Sindbis
Snowshoe hare
West Nile

c. Testing of rabbit sera for optimal dilution to use in ELISA:

The ammonium sulfate concentrates of rabbit IgG were tested at several dilutions, usually 1:200, 1:1000, 1:4000, 1:16000, and 1:64000 dilutions. Antigens were used in most cases at 1:10 dilution. There was considerable variation in antibody response of individual rabbits. The results of those antigen-antibody pairs that functioned well are shown below.

Antigen-antibody	Antigen dilution	Optimum IgG titer	Volume ml
Bussuquara	1:25	1:200	28
		1:400	15
		1:2000	40
		1:16000	34
		1:32000	10
Bwamba	1:10	1:500	15
Chandipura	1:10	1:500	27
		1:800	47
		1:4000	43
		1:32000	87
Cocal	1:10	1:8000	6
		1:16000	80
		1:32000	96
Germiston	1:10	1:1000	37
		1:16000	38
Hazara	1:10	1:500	15
		1:64000	42
Ilheus	1:10	1:64000	18
Jamestown Canyon	1:10	1:16000	62
		1:32000	30
Japanese encephalitis	1:10	1:1000	33
		1:64000	13
Mucambo	1:10	1:400	13
		1:500	76
		1:4000	27
		1:32000	40
Quaranfil	1:10	1:4000	85
		1:8000	95
Semliki Forest	1:10	1:32000	35
		1:64000	40
Sindbis	1:10	1:4000	30
		1:16000	30
		1:32000	38
Snowshoe hare	1:10	1:500	73
West Nile	1:10	1:32000	15
		1:64000	40
		1:128000	50

6. DISCUSSION AND CONCLUSIONS

The major effort of this project was the production of sucrose-acetone extracted mouse brain antigens for 54 viruses. This was for the most part accomplished. Not all of the attempts to immunize rabbits were successful. Those antigens for which a companion rabbit serum is not available will be excellent reagents to use in IgM capture tests of early convalescent sera to make a presumptive diagnosis. They are also highly useful in ELISA with conventional mouse IgG as the capture antibody. Further, many of them can be used in the hemagglutination-inhibition test.

As detailed in the Annual Reports, the project underwent an extensive delay when the only technician, an experienced worker, developed colon cancer. He was away for six months of sick leave and back vacation with full pay. In spite of this, after extensions without additional funds, new personnel were trained and it was possible to a considerable degree to catch up. The final antigens and antibodies should prove to be extremely useful diagnostic reagents.

7. PUBLICATIONS

None.

8. PERSONNEL RECEIVING CONTRACT SUPPORT

Robert E. Shope, M.D.	Professor of Epidemiology, P.I.
James Washington	Technician A, April 1987 through October 1988
Kaveh Khoshnood	Associate in Research, July through December 1989
Shirley J. Tirrell, MPH	Associate in Research, January 1990 through March, 1991

All financial records pertaining to this contract are subject to audit review.